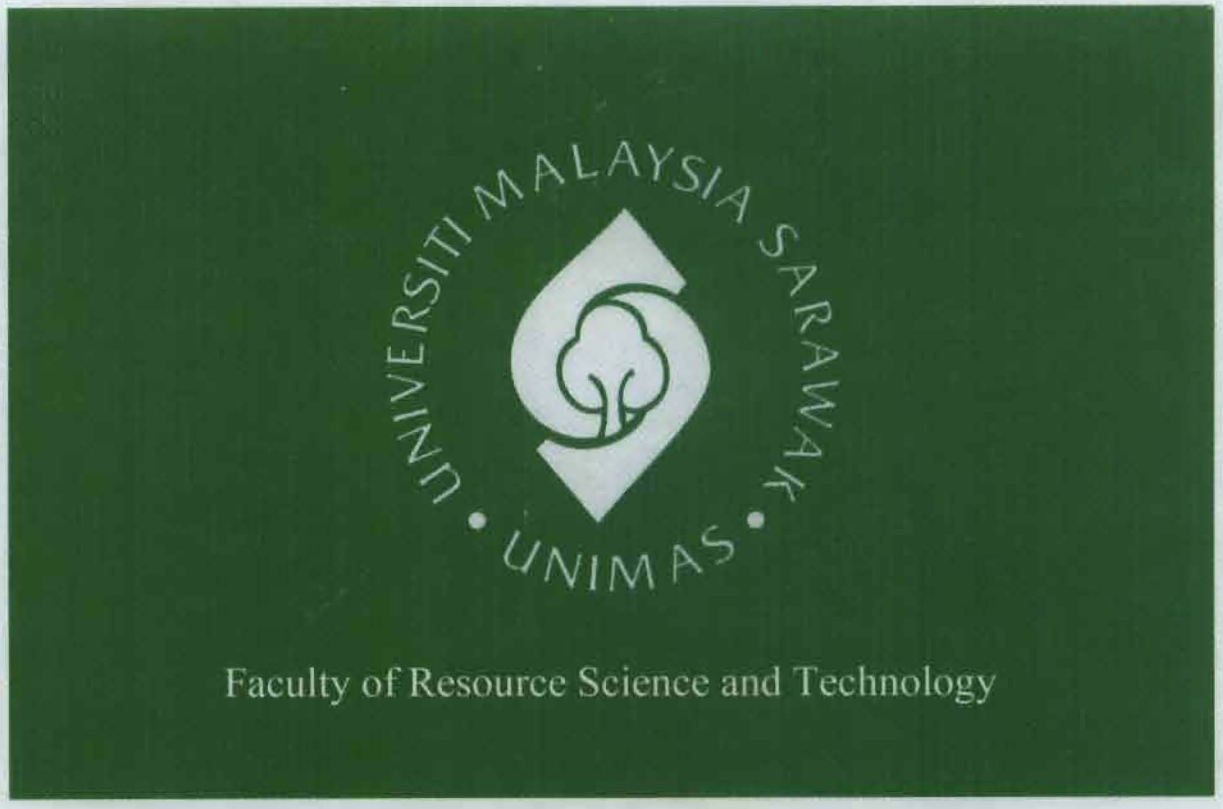

The use of Loop AMP in Detection of Shiga-toxin (*stx*) Gene among *Escherichia coli* from Aquaculture and Other Environments

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Bachelor of Science with Honours
(Resource Biotechnology)
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(26037)

This project is submitted in partial fulfilment of the requirements for the degree of
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Declaration

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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List of Abbreviation

AGE	Agarose Gel Electrophoresis
BIP	Backward Inner Primer
CTAB	Hexadecyltrimethylammonium bromide
DMBA	p-dimethylaminobenzaldehyde
E.coli	*Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FIP	Forward Inner Primer
GUD	β-glucuronidase
HC	Hemorrhagic Colitis
HUS	Hemolytic Uremic Syndrome
LAMP	Loop-mediated Isothermal Amplification Method
LB	Luria Bertani
EMB	Eosin Methylene Blue
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
SDS	Sodium Docecyl Sulphate
STEC	Shiga-toxin producing *Escherichia coli*
*stx*1	Shiga toxin 1
*stx*2	Shiga toxin 2
TAE	Tris-acetic acid
TBE	Tris-boric acid
TTP	Thrombotic Thrombocytic Purpura
UV light	Ultra Violet light

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ABSTRACT

Considerable amount of cases of Shiga-toxin producing *Escherichia coli* (STEC) has been reported which result in the need to detect the presence of this harmful *E. coli* in food and environmental sources. Diseases caused by STEC are Hemolytic Uremic Syndrome (HUS) and Hemorrhagic Colitis (HC). A lot of samples being used in researches are mostly from retailed chain store or farms. The need to look into aquaculture and other environmental water is very crucial since there are very few researches done on aquaculture and other environmental water. In this study, samples were taken from Asajaya Empurau fish farm, Damai river stream, Permai beach and Permai rainforest resort. Enterobacteriaceae were successfully isolated using Eosin Methylene Blue agar and *E. coli* were successfully identified through a series of biochemical test. DNA was isolated using boiling method and Loop-AMP was conducted to detect toxigenic genes carried by *E. coli*. It was found that none of the isolates were carried *stx* 1 or *stx* 2 gene. However, multiplex-PCR detected three isolates which carried *stx* 1 and *stx* 2 gene. $(GTG)_5$-PCR was carried out to detect the diversity among the isolates and was found that the genetic diversity among *E. coli* isolates were not high. Two major clusters with one of the cluster having 2 sub clusters were detected. Water quality for Damai downstream, Damai middle stream, Permai beach, Permai rainforest resort were classed at class IIB which is safe for water recreational activities with body contact.

Keywords: *Escherichia coli*, Biochemical test, Loop-AMP, *stx* gene, $(GTG)_5$-PCR.

ABSTRAK

Sejumlah kes besar yang dilaporkan mengenai pengesahan Escherichia coli yanf menghasilkan Shiga-toxin (STEC) telah dikaitkan dengan makanan dan persekitaran. Penyakit-penyakit yang disebabkan oleh STEC adalah Hemolytic Uremic Syndrome (HUS) dan Hemorrhagic Colitis (HC). Banyak sample yang diuji adalah daripada rantaian kedai makanan atau kebun. Keperluan untuk menguji akuakultur air dan air daripada persekitaran lain adalah penting kerana kurang penyelidikan dalam bidang ini. Dalam kajian ini, sample telah diambil daripada sungai Damai, pantai Permai dan Permai rainforest resort. Enterobacteriaceae telah berjaya diasingkan dengan menggunakan EMB agar dan E. coli telah berjaya dikenalpasti dengan meggunakan ujian biokimia. Pengesahan toxigenic gene daripada E. coli menggunakan Loop-AMP telah menunjukkan tiada toxicgenic gene yang dijumpai. Tetapi, terdapat tiga isolat yang menunjukkan keputusan positif menggunakan multiplex-PCR. $(GTG)_5$-PCR telah dijalankan untuk mengesan kepelbagaian antara isolat-isolat dan didapati kepelbagaian genetik antara E. coli adalah tidak tinggi. Dua kluster utama telah dikesan dengan salah satu kluster mempunyai 2 kluster kecil.Kualiti air Damai downstream, Damai middle stream, pantai Permai dan Permai rainforest resort merupakan air kelas IIB iaitu sesuai untuk aktiviti air dengan sentuhan badan.

Kata kunci: *Escherichia coli, ujian biokimia, Loop-AMP, multiplex-PCR, $(GTG)_5$-PCR.*

1.0 Introduction

Escherichia coli are normally known as a non-pathogen and also beneficial to human intestinal tracts. However, there is certain pathogenic *E. coli* which is responsible for the outbreak of *E. coli* diseases in Germany (Malaysia Times, 2009) in year 2011 and other minor cases in United States. Although most cases of *E. coli* outbreaks are associated with raw meat and unpasteurized milk (Griffin, 1991), and the recent outbreak in Germany which is associated with vegetables (Dempsey & Neuman, 2011), all these sources came from water. Currently, there are very few reports and researches on the presence of STEC in environmental water and mostly on marketed beef in West Malaysia (Radu *et al.,* 1998).

The detection of toxigenic genes (*stx*1 and *stx2*) of *E. coli* from environmental water is utmost important because environmental water is the root of cause for the appearance of STEC in farm animals and vegetations. This study was proposed to detect presence of toxigenic *E. coli* in environmental water.

Loop-mediated isothermal amplification (LAMP) is a novel technique developed by Notomi *et al.*, (2000) which has a high sensitivity and accuracy in replicating high amount of DNA in isothermal conditions. LAMP utilizes 2 inner primers, 2 outer primers and a DNA polymerase. The 2 inner primers are called Forward Inner Primer (FIP) and Backward Inner Primer (BIP) which is complementary to the sense and anti-sense sequences of the target DNA. Two outer primers, F3 and B3 are designed to release strand displacement DNA. Due to the used of 4 primers, sensitivity of LAMP is very high and presence of amplified product generally indicates targeted genes being amplified.

Sahilah *et al.* (2010) had done researches on detection of *stx*1 gene from eggs and imported beef meats. Their research has shown positive result for the presence of *stx* genes. However, limited research has been done on environmental water in Malaysia.

Elsewhere, most of the studies done are on animal stools and foods (Rodolpho & Marin, 2007 ; Adwan *et al.*, 2002; Keen *et al.*, 2006 ; Adwan & Adwan, 2004), most of them utilize conventional PCR method. Very few studies has been done using environmental water and aquaculture water (Kuhnert, Boerlin & Frey, 2000 ; EL-Jakee *et al.*, 2009) and even few researches utilize Loop-mediated isothermal amplification method. The efficiency of LAMP is being compared with multiplex-PCR where time require to complete the reaction is concerned.

The main objectives of this project were to:

a) isolate *E. coli* from aquaculture and other environmental water.

b) rapidly detect toxigenic genes in *E. coli* using Loop Mediated Isothermal Amplification Method (LAMP)

c) characterize *E. coli* isolates using $(GTG)_5$-PCR fingerprinting analysis and,

d) to assess the water quality of water level of different sampling location.

2.0 Literature Review

2.1 *Escherichia coli*

Escherichia coli are gram negative, rod shape bacterium under the family of enterobacteriaceae. *E. coli* are mainly found in the intestinal tract of mammalians. Most of them are not hazardous but in fact, beneficial to their host. However, there are small portions of *E. coli* which can cause diseases and STEC O157:H7 *E. coli* was identified when two observations were made by Andrew and Growther (2011). The first observations would be bloody diarrhea and severe abdominal cramps which is termed as hemorrhagic colitis (HC). The second observation was made by Karmali *et al.* as cited in Andrew and Growther (2011) where there have been irregular reports on HUS with fecal cytotoxin and also *E. coli* in stools. *E. coli* that cause diarrhea can be distributed into four common categories which are enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), and enterohemorrhagic (EHEC). All these groups have to be sorted according to their serotypes and pathogenesis (Levine, 1987).

2.2 Shiga toxin producing *Escherichia coli* (STEC)

Classification of *E. coli* is based on their virulence factor (Fairbrother & Nadeau, 2006). These pathogenic strains of *E. coli* are called Shiga-like toxin producing *E. coli* (STEC) and can produce potentially harmful toxin called Shiga toxin 1 and 2 (*stx*1 and *stx*2) because the toxins are similar to toxins produced by *Shigella dysentriae.* STEC is also known as Verotoxin producing *E. coli* (VTEC) as the toxin (*vtx1* and *vtx2*) produced acted on Vero cell. The function of *stx1* and *stx2* is to disrupt the proteins synthesis in host.

E. coli O154:H7 is one of the strains of *E. coli* that can cause infection towards human. *E. coli* O157:H7 can cause Hemorrhagic Colitis (HC) followed by Hemolytic Uremic Syndrome (HUS) which occurred mostly in children (Bidet *et al.*, 2005). Hemorrhagic Colitis (HC) is characterized by bloody diarrhea, abdominal cramps, inflammation of large bowels and vomiting (Cressey *et al.*, 2007). HUS followed HC which is characterized by the onset of low platelet count and renal injury and death which include seizures and coma (Washington State Department of Health, 2009).

The main reservoir for STEC is cattle, in particular, and also other ruminants (O'Sullivan *et al.*, 2007). Raw meat and unpasteurized milk is also one of the sources for STEC according to Griffin (1991). The route of transmission for STEC is through contact with water while swimming in contaminated lakes or pools. Transmission also occurs through person to person and direct contact with animal fecal through recreational activities (O'Sullivan *et al.*, 2007).

Enterohemorrhagic *E. coli* (EHEC) is the subset of STEC because EHEC produces Shiga toxin. Intimin, a protein encoded by the gene *eae* which gives EHEC the abilities to form A/E lesion in intestinal wall by disrupting the brush border microvilli (O'Sullivan *et al.*, 2007). The gene is located at Locus of Enterocyte Effacement (LEE). EHEC is also one of the main concern in the outbreaks of HC and HUS (Brugere *et al* ., 2004).

2.3 Hemorrhagic Colitis (HC)

Hemorrhagic Colitis (HC) was first discovered by Riley *et al.* (1983) where there was unusual gastrointestinal illness that causes abdominal cramp, watery diarrhea followed by bloody diarrhea. Fever is usually not accompanied but some may have slightly higher in body

temperature. Symptoms which usually indicate that one is having HC are bloody diarrhea several days after watery diarrhea accompanied with vomiting (Cohen & Giannella, 1992). Very young and old individuals are more prone towards the infection. Hemolytic Uremic Syndrome (HUS) is a severe complication that comes from HC. Around 2 to 7% of HC patients will develop, according to Boyce (2007).

2.4 Hemolytic Uremic Syndrome (HUS)

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2.5 Eosin Methylene Blue (EMB) agar

Eosin methylene agar was first described by Harris which is a selective agar for gram-negative bacteria (Levine, 1918). The constituent of EMB agar consists of peptone, lactose, sucrose, eosin Y dye and methylene blue. The methylene blue is used to inhibit the growth of most gram-positive bacteria (Leininger *et al.*, 2001). Lactose fermenting bacteria can produce acidic products which will cause the eosin to change colors from colorless to black (American Society

for Microbiology, 2010). Normally, fecal and non-fecal coliforms are encouraged to grow with the presence of lactose and sucrose. *E. coli* which has vigorous lactose or sucrose fermenting abilities will produce green metallic sheen on the agar.

2.6 Biochemical test

Tests for Indole, motility and production of H_2S are carried at the same time using an agar called S.I.M agar. Indole test was used back in 1889 to distinguish *E. coli* from *Enterobacter aerogenes* (Isenberg & Sundheim, 1958). The purpose of indole test is to test the ability of degrade amino acid tryptophan. Any bacteria that have the enzyme tryptophanase will be able to give a positive result. When tryptophan is deaminade and hydrolysed, indole will be produced and a chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) will produce red coloration (rosindole) under acidic condition. Sodium Thiosulfate is included in S.I.M agar and bacteria can utilize this component to produce hydrogen sulfide (H_2S). Since H_2S is colorless, the inclusion of ferric ammonium will react with H_2S and produce black precipitation. Motility of bacterium is assessed by the growth pattern of bacteria. If the growth of bacterium is along the stab line, then it means that the bacterium is not motile. If the stab line is not clearly visible and the growth of bacterium is spread sideways along the stab line, then the bacterium is motile.

Methyl Red (MR) test is to test for the production of acid which is the result of consumption of glucose. Methyl red reagent is a pH indicator and an acidic medium will turns the methyl red reagents into red color. Voges-Proskaeur (VP) test is also a protocol that tests for the consumption of glucose but with another end product. VP test is to test for the production of a neutral end product, acetoin. VP A reagent which contains naphthol and VP B reagent which

contains Potassium Hydroxide (KOH) are used in VP test. The amount and the order of the reagent added must be correct for the end result to show.

Citrate test is carried out to test for organisms that use only citrate as the only carbon source. Bromothymol Blue which is a pH indicator is included in the agar and if the organism is able to used citrate as the carbon source and produce alkaline end products, then blue coloration will be formed.

Triple Sugar Iron (TSI) agar test is to test for the gram-negative bacteria of utilizing either glucose and lactose or sucrose in fermentation (Washington, 1981). The TSI agar contains 10 parts lactose, 10 parts sucrose and 1 part sucrose. Phenol red which is a pH indicator is also presence in the agar which changes to yellow (indicate production of acid) or blue (indicate production of alkaline product). Sodium thiosulfate and ferrous ammonium sulfate also presence in the agar as an indicator for the presence of H_2S production. Oxidase test is carried out to determine whether a microorganism contains a cytochrome c oxidase system. Colony is picked and rub on filter paper dipped in oxidase solution. Blue coloration indicate positive while no color change indicate a negative result.

2.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis (AGE) is the most common and cost benefit way to check for the presence and weight of DNA. Ethidium Bromide (EtBr) is a common stain used in AGE for detection of DNA, it moves into the spaces of the base pairs of the double helix. EtBr absorbs UV light and re-emits them, that is why a UV transilluminator is used (Burns, 1969). There are 2 types of staining for EtBr, which are pre-staining and post-staining. Pre-staining is where EtBr is

added to the warm agarose before pouring onto the casting tray. Pre-staining is performed to gives a better resolution but chances is that there can be contamination on the casting tray. Etbr will also incorporate into the DNA and increase the weight of the DNA, thus slows down the movement of DNA moving through the gel. By soaking the agarose gel in EtBr and buffer solution after electrophoresis, this process is call post-staining. Post-staining is performed if we do not want our tank to be contaminated.

TBE buffer, also known as Tris/Borate/EDTA buffer is a mixture of Tris base, boric acid and EDTA in a certain amount. The differences between TAE and TBE is that instead of using boric acid, TAE substitute boric acid with acetic acid. Experiment done by Kato, Miura, and Wake (1999), showed that TBE is capable of showing a better resolution for smaller DNA fragments than TAE.

2.8 Loop-mediated Isothermal Amplification Method

LAMP is a novel method invented in year 2000 by Notomi *et al.* (2000). LAMP differ with other amplification method by having high sensitivity and efficiency when amplifying nucleic acid. Operating under just one temperature is one of the main features that cause LAMP to be so widely accepted because simple apparatus like water bath which is, both easily accessible and cost effective, the only apparatus needed. As compare to conventional PCR, a machine with temperature preset is needed so that denature and re-nature can occur simultaneously. Not to mention the cost needed to maintain a PCR machine.

LAMP uses four primers, 2 inner primers and 2 outer primers. The inner primers are term Forward Inner Primer (FIP) and Backward Inner Primer (BIP) which anneal to the sense and anti-sense of the target DNA. F2c and B2 are designed inside the target DNA sequence. Two

inner sequences, F1c and B1 are designed inside F2c and B2 and two outer sequences F3c and B3 are designed outside F2c and B2 respectively. So from 5', we have B3 , B2 , B1 , F1c , F2c , F3c. This will be our targeted DNA sequences. FIP is designed so that it will anneal to the F2c of the targeted DNA strand. Therefore, sequence of FIP is F1c, TTTT spacer and F2. Similarly, BIP sequence would be B1c, TTTT spacer and B2. The 2 outer primers would be F3 and B3 which corresponds to F3c and B3c respectively.

At the start of the experiment, all four primers are used but in the later stage of the amplification, only the inner primers are used (Notomi *et al.*, 2000). Initially, FIP anneals to F2 and strands are being synthesized. Later, F3 which is lower in concentration will starts to anneal to F3c and strands displacement occurred. The displaced strand contains F1c and F1 which is complementary to each other and forms a loop. BIP then anneal to the displaced strands and form complementary strand, B3 would then anneal to B3c and form a new displaced strand. This new displaced strand will have B1c and B1 and F1c and F1 at each ends, they will then form loop and this is the initial product for LAMP. The amplification starting from this point onwards only uses the 2 inner primers.

A research done by Nagamine *et al.* (2002) has stated that the use of loop primer can effectively decrease the time require by one-third to one-half. Loop primers (Loop Primer F or Loop Primer B) are complementary to the space between B1 and B2 or between F1 and F2. Therefore, while FIP is hybridizing F2c region, Loop Primer B also starts the hybridization on the other end. This is how time is save using Loop primers.

3.0 Materials and Methods

3.1 Sampling

Water and sediment samples were sampled on weekdays and weekend. Three sample sites were chosen along Damai river which were upper stream, middle stream and downstream. As for Permai Rainforest Resort, 3 samples sites were also collected which were the inlet, middle and outlet. One sample site was chosen for Permai Beach. Samples were also collected from Asajaya fish farm (Empurau fish) ; the 3 types of pond which were culture pond, breed pond and sale pond. Each pond has inlet, middle and outlet. Homogenization was carried out for each pond.

3.2 Revival of sample isolates

One hundred micro liter of glycerol stock solution was pipetted into Luria Bertani broth and incubated at 37°C for overnight. The bacteria culture was then plated on EMB agar to check for purity.

3.3 Confirmation of positive strains EDL933

The positive EDL 933 strains was revived in Luria Bertani's broth for overnight at 37°C and after revival, EDL 933 strains was streaked onto EMB agar and incubated at 37°C for overnight. After incubation, single colony of positive result (green metallic sheen) was picked and inoculated into Luria Bertani's broth. The LB broth was incubated at 37°C for overnight and Sulfide-Indole-Motility (S.I.M) test was carried out using the overnight bacteria broth. The culture was stabbed into the S.I.M agar and incubated at 30°C for 24 hours. After incubation, production of H_2S was determined by the production of black precipitation. Next, motility was determined by the

growth of the bacteria. If the bacteria grow on the stabbing line, then it is non-motile. If the stab line diffused into the agar, then the bacteria is motile. Lastly, few drops (6-8 drops) of Kovac's reagent were added to the S.I.M agar. Red layer formed indicate the bacteria is indole positive. Yellow or brown layer is for negative result.

3.4 Sample Processing

Serial dilution were prepared from 10^1 to 10^5 for sediment samples and serial dilution from 10^1 to 10^3 were picked for water samples. Each dilutions were spread plated on both NA and EMB agar with 2 replicates. Both replicates of NA and EMB agar were incubated at 37°C for 24 hours and plate counting were performed. Plates with count of 30-300 colonies were selected and inoculated into LB broth and incubated at 37°C for 24 hours. After 24 hours, DNA extraction was carried out.

3.5 Biochemical Test

The culture was stabbed into the S.I.M agar and incubated at 30°C for 24 hours. After incubation, production of H_2S will be determined by the production of black precipitation. Next, motility was determined by the growth of the bacteria. If the bacteria were grown on the stabbing line, then it is non-motile. If the stab line diffused into the agar, then the bacteria were motile. Lastly, few drops (6-8 drops) of Kovac's reagent were added to the S.I.M agar. Red layer form indicated that the bacteria were indole positive. Yellow or brown layer was the negative result. Isolates showing positive result would be tested with Triple Sugar Iron (TSI) agar test.

As for citrate test, the cultures were streaked on citrate agar and incubated at 65°C for 24 hours. A loopful of bacteria culture were inoculated into 40ml of MR-VP broth and incubated at 37°C for 48 hours. After incubation, 20ml of the broth would be transferred into another sterile tube and tests for both Methyl Red and Voges-Proskauer were carried out. For Methyl Red test, 6 to 8 drops of Methyl Red reagents were added. For Voges-Proskauer test, 12 drops of VP A reagents were added first followed by 4 drops of VP B reagents. The tubes were left on bench for at least 30 minutes. Isolates with positive indole and methyl red test were further tested with TSI agar test and oxidase test.

3.6 Preparation of Stock Culture

Isolates which showed same characteristic with EDL933 in the biochemical tests were selected and inoculated into LB broth and incubated at 37°C for 24 hours for enrichment. After 24 hours, the isolates were streaked onto EMB agar at 37°C for 24 hours. One pure colony from each incubated EMB agar was then streaked onto LB slant agar. The LB slant agar was then stored at 4°C. Each time the isolates were required, colonies from slant agar were streaked onto LB agar plate and incubated at 37°C for 24 hours and after that one colony was picked and inoculated into LB broth.

3.7 DNA extraction (Boiling method)

The overnight bacteria culture was used for DNA extraction. One thousand and five hundred micro liters of overnight bacteria culture was transferred into 1.5 ml microcentrifuge tube. The

microcentrifuge tube was centrifuged at 10,000 rpm for 5 minutes. Supernatant was discarded and the steps above were repeated for one more time. After the supernatant was discarded for the second time, 500µl of ddH_2O was added and vortexed to resuspend the cell pellet. The microcentrifuge tube was then boiled for 10 minutes and immediately placed on ice for 5 minutes after boiling. After 5 minutes, the microcentrifuge tubes were sent for centrifugation at 10,000 rpm for 10 minutes and the supernatant was collected.

3.8 Multiplex-Polymerase Chain Reaction

Presumptive *E. coli* from biochemical test were selected for multiplex-PCR analysis. The component of multiplex-PCR used in this project was listed in the Table 1. A positive control (EDL 933 strain) and negative control were included. Table 2 shows the temperature and number of cycles for multiplex-PCR and Table 3 shows the primer sequences for *stx* 1 and *stx* 2.

Table 1 : Multiplex-PCR components and volume.

PCR components	Volume (µl)
5X buffer	3.0
25mM $MgCl_2$	1.5
25mM dNTPs	1.0
10µm/nmol primer	
Stx 1 F	0.5
Stx 1 R	0.5
Stx 2 F	0.5
Stx 2 R	0.5
DH_2O	12.1
DNA template	5.0
Taq polymerase	0.4
Total	25

Table 2 : The temperature cycling for Multiplex-PCR analysis.

Step	Temperature (°C)	Time (min)	
Pre-denaturation	95°C	5	
Denaturation	94°C	1	35 cycles
Annealing	58°C	1	
Extension	72°C	1	
Final Extension	72°C	10	

Table 3 : Primer sequence for *stx* 1 and *stx* 2

Gene name	Primer sequence	Control Strain
Stx 1 F	5' ATA AAT CGC CAT TCG TTG ACT AC 3'	EDL 933
Stx 1 R	5' AGA ACG CCC ACT GAG ATC ATC 3'	EDL 933
Stx 2 F	5' TTA ACC ACA CCC CAC CGG GCA GT 3'	EDL 933
Stx 2 R	5' GGA TAT TCT CCC CAC TCT GAC ACC 3'	EDL 933

3.9 Agarose Gel Electrophoresis

One percent agarose agar was prepared by mixing 0.5g of agarose powder with 50 ml of TBE buffer. EtBr was added before melting the agarose powder under microwave oven. The melted agarose solution was placed on cold water to reduce the temperature and cast onto the casting tray. After agarose gel has hardened, ladder and PCR product were inserted into the well and run at 80 volts for 35 mins. After the gel was ran, the agarose gel was brought to dark room for viewing.

3.10 Loop-mediated isothermal amplification (LAMP)

Two µl of DNA was added into the LAMP reaction mixture and incubated at 65°C for 60 mins followed by 80°C for 2 mins to terminate the reaction. Table 4 shows the recipe for LAMP reaction mixture with slight modification.